Short communication: The preruminant calf as a model for characterizing the effects of vitamin D status in the neonate[1]

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ABSTRACT

The objective of this study was to evaluate the feasibility of using the preruminant dairy calf as a model for evaluating effects of vitamin D status in the neonate. Because the newborn calf can be sustained during the first weeks of life solely on a fluid diet having a defined composition, has documented nutritional requirements, and is minimally affected by repeated samplings of peripheral blood, it has the potential to serve as a model for characterizing nutrient-specific effects on the growth and health of the neonate. Colostrum-fed Holstein bull calves (n = 13) entered the trial at approximately 4 d of age. All calves were fed a custom-formulated milk replacer devoid of vitamin D. Plasma 25-hydroxyvitamin D_3 concentrations in all calves were determined on a regular basis beginning at d 0. Using this information, low- and high-status groups of calves were established by subcutaneous administration of 25-hydroxyvitamin D_3. To maintain targeted plasma 25-hydroxyvitamin D_3 concentrations in low (<30 ng/mL) and high (>60 ng/mL) vitamin D–status calves, low-status calves (n = 6) received a total of 8,600 IU (2,225 IU/wk) of vitamin D during the experimental period and high-status calves (n = 7) received 54,000 IU (13,500 IU/wk). Concentrations of 25-hydroxyvitamin D_3 in low-status calves averaged 27 ng/mL, compared with 78 ng/mL in high-status calves, and were less at all sampling times from d 7 to d 28. Concentrations of 1,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3 were not correlated. Calcium, magnesium, and phosphorous concentrations were unaffected by 25-hydroxyvitamin D_3 administration; however, plasma calcium and 1,25-dihydroxyvitamin D_3 concentrations were correlated. Calcium and magnesium concentrations decreased with age but remained within normal ranges for dairy cattle. These results indicate that it is possible to predictably control vitamin D status over a 28-d period and suggest that the preruminant calf might be useful as a model for studying effects of vitamin D on growth, development, and immune function in the neonate.

Key words: preruminant calf, vitamin D status, neonatal nutrition

Vitamin D plays a pivotal role in calcium homeostasis, cell differentiation and proliferation, and innate and adaptive immune function (van Etten et al., 2008). Vitamin D is derived from 2 sources: dietary intake [vitamin D_2 (ergocalciferol) from plant sources or D_3 (cholecalciferol) from animal sources] and the conversion of 7-dehydrocholesterol to cholecalciferol in the skin, a reaction catalyzed by UV light. In the absence of sufficient sun exposure for dermal conversion, vitamin D becomes an essential nutrient. At body temperature, cholecalciferol spontaneously converts to vitamin D_3. Vitamin D provided in the diet or produced in the skin is rapidly transported to and sequestered by the liver, where it is available for conversion to 25-hydroxyvitamin D [$\mathbf{25(OH)D_3}$]. This metabolite in association with vitamin D binding protein is the predominant circulating form and is considered the best indicator of vitamin D status (Horst et al., 1994; Looker et al., 2008). In hypocalcemic states, $25(OH)D_3$ is hydroxylated to 1,25-dihydroxyvitamin D_3 [$\mathbf{1,25(OH)_2D_3}$] in the kidney. As the major mediator of biological activity of vitamin D, this metabolite acts to increase active transport of calcium and phosphorous across intestinal epithelium and potentiates the action of parathyroid hormone to increase bone calcium resorption.

Historically, vitamin D deficiency has been associated with osteomalacia, osteopenia, osteoporosis, and muscle weakness. More recently, deficiency in humans has been linked to increased risk of cancer, autoimmune disease, cardiovascular disease, and infectious disease (Holick, 2007; Norman, 2008). In humans, normal circulating concentrations have been defined as 22 to 30 ng/mL for $25(OH)D_3$ and 25 to 34 pg/mL for $1,25(OH)_2D_3$ (Salle et al., 2000). However, recent evidence suggests that in humans, greater plasma $25(OH)D_3$ concentrations

might be necessary to ensure sufficiency (Hollis, 2005). Plasma $25(OH)D_3$ at >32 ng/mL enhances innate immunity in humans (Holick, 2007), a level exceeding the amount necessary to ensure calcium homeostasis. In healthy dairy cows, plasma $25(OH)D_3$ concentrations range from 20 to 50 ng/mL. Values less than 5 ng/mL are considered indicative of vitamin D deficiency, whereas concentrations in excess of 200 to 300 ng/mL are indicative of vitamin D toxicosis (Horst et al., 1994). It is not clear if these adult values apply to the newborn calf.

The current recommended vitamin D requirement (NRC, 2001) for preruminant calves is 318 IU/d for a 45-kg calf fed 0.53 kg of DM/d. Commercial milk replacers typically provide the calf 2,650 IU/d, substantially more vitamin D than the NRC recommendation. Justification for the current NRC recommendation or evidence supporting the substantially greater amounts in commercial milk replacers is limited. In addition, data regarding cutoffs of plasma $25(OH)D_3$ that define the lower limit of adequacy or sufficiency in the preruminant calf are sparse. Given recent evidence in other species suggesting vitamin D concentrations not previously considered representative of deficiency might be linked to nonskeletal diseases (Holick, 2007), reexamination of the effects of vitamin D supplementation on the neonate might be warranted.

The purpose of the current study was to develop a method for manipulating, in consistent fashion, the vitamin D status of the milk replacer–fed calf. Successful demonstration of this approach would not only yield new information regarding vitamin D status in the preruminant calf but also might provide a suitable animal model for evaluating the effects of vitamin D on the growth, development, and health of the neonate.

Animal-related procedures were approved by the Animal Care and Use Committee of the National Animal Disease Center (NADC), Ames, Iowa. Holstein bull calves (n = 13) from a single Wisconsin dairy herd were given 4 L of colostrum within the first 6 h after birth. Navels were dipped in iodine, and an *Escherichia coli* vaccine (Genecol-99, Schering-Plough Animal Health, Union, NJ) was administered orally at birth. At less than 4 d of age, calves were transported to NADC where they were housed individually in elevated pens (1.52 m long × 0.92 m wide × 0.92 m high) in a calf-specific barn with temperatures maintained between 15 and 18°C. Each calf was given 2 mL of iron dextran (100 mg/mL, AmTech, Phoenix Scientific, Inc., St. Joseph, MO) intramuscularly, a 2.5-mL solution of sodium selenite (2.19 mg/mL) and α-tocopherol (50 mg/mL; BoSe, Schering-Plough Animal Health) intramuscularly, and 2 mL of a vitamin B complex (Phoenix Scientific, Inc.) subcutaneously. At the time of arrival, calves were of-

Table 1. Composition of milk replacer[1] fed to all calves during the 28-d experimental period

Component	Unit	Analysis
CP	% of DM	28.09
Fat	% of DM	20.05
Lactose	% of DM	30.00
Vitamin D_3	IU/kg	0.00
Vitamin A	IU/kg	13,608
Vitamin E	IU/kg	90.80
Calcium	% of DM	0.75
Phosphorous	% of DM	0.06
Magnesium	% of DM	0.11

[1]Manufactured by Animix LCC, Juneau, WI. Not all components are listed.

fered Enterolyte (Pfizer, Exton, PA) mixed in warm water following the manufacturer's directions.

Within 24 h after arrival, calves were weighed and assigned randomly to 2 treatment groups that would represent low [15–25 ng of $25(OH)D_3$/mL of plasma, n = 6] and high vitamin D status [55–65 ng of $25(OH)D_3$/mL of plasma, n = 7]. During the 28-d study, all calves were fed twice daily 0.34 kg of a 28% CP, 20% fat, all-milk protein milk replacer (Table 1, Animix LLC, Juneau, WI) devoid of exogenous vitamin D and reconstituted to 15% dry matter. Plasma $25(OH)D_3$ concentrations in all calves were determined at regular intervals, beginning at d 0. Based on these data and calf BW, low- and high-status groups of calves were established by subcutaneous administration of $25(OH)D_3$ (range: 75 to 500 mg/mL in reagent-grade ethanol, 1 mL total/administration). Low-status calves received, on average, 8,600 IU of vitamin D during the experimental period, and high-status calves received 54,000 IU.

Concentrations of $25(OH)D_3$ and $1,25(OH)_2D_3$ in plasma and milk replacer were quantified using radioimmunoassay as described by Hollis et al. (1993 and 1996, respectively). Serum calcium and magnesium concentrations were determined by atomic absorption spectrometry (Perkin-Elmer Corp, Norwalk, CT) as described previously (Waldron et al., 2003). Serum phosphorous (PO_4) was measured colorimetrically using a procedure modified from Parekh and Jung (1970) and validated in our laboratory. Briefly, serum (125 µL) was precipitated by acidification with 1 mL of molybdic-trichloroacetic acid solution. After 5 min, the solution was centrifuged (1,000 × g), and 100 µL of supernatant was transferred in duplicate to 96-well microplates (Costar, Corning Inc., Acton, MA). Finally, 150 µL of p-phenylenediamine reagent (1 g of p-phenylenediamine dihydrochloride in 100 mL of 5% $Na_2S_2O_5$ solution) was added to all wells. Plates were incubated for 20 min at room temperature. Phosphorous was determined at a wavelength between 690 and 560 nm using a Thermo

Max tunable, microplate-reader spectrophotometer (Molecular Devices, Sunnyvale, CA).

Data used in the final analyses were from vitamin D and mineral assays performed en bloc at the conclusion of the study. Data were analyzed as a completely randomized design using Statview software (version 5.0, SAS Institute, Inc., Cary, NC). Calf served as the experimental unit in the analysis of all data. Vitamin and mineral concentrations were analyzed as a split-plot with repeated-measures ANOVA. The model included the fixed effects of treatments, time (day after initiation of treatments), and treatment × time interaction, with calf included in the model as the random effect. Fisher's protected least significant difference was applied when treatment, time, or treatment × time effects ($P < 0.05$) were detected by the model. Pearson product–moment correlations were computed between plasma concentrations of $25(OH)D_3$, $1,25(OH)_2D_3$, Ca, PO_4, and Mg. Correlations were judged significant at $P < 0.01$ with r >0.40.

Results demonstrate that by adjusting the amount of $25(OH)D_3$ administered subcutaneously using data from periodic analysis of plasma $25(OH)D_3$ concentrations, it is possible to establish groups of calves representing low and high vitamin D status (Figure 1a). Parenteral administration of vitamin D provides a degree of precision not afforded by feeding milk replacer with varying amounts of vitamin D. Using this approach, treatment ($P < 0.001$) and time ($P < 0.0001$) effects, as well as treatment × time interaction ($P < 0.01$), were significant for plasma $25(OH)D_3$ concentrations. At the beginning of the trial (d 0), $25(OH)D_3$ concentrations in both groups were not different ($P > 0.05$) and averaged 25 ng/mL, a value comparable to $25(OH)D_3$ concentrations observed in newborn colostrum-fed and colostrum-deprived calves sampled 7 h after birth (Rajaraman et al., 1997). The $25(OH)D_3$ concentrations in the high-status group increased ($P < 0.05$) between d 0 and d 7 and were greater ($P < 0.05$) than that in the low-status group from d 7 through d 28. On d 7, 21, 25, and 28, concentrations exceeded 90 ng/mL, more than 3-fold greater than concentrations in low-status calves, and exceeded the maximal concentrations observed in standard (87 ng/mL) or intensified (89 ng/mL) milk replacer–fed calves (Nonnecke et al., 2007). These concentrations were achieved by administering, on average, a total of 54,000 IU of vitamin D/calf during the 28-d study. In contrast, $25(OH)D_3$ concentrations in the low-status group did not change significantly from d 0 to d 25 and ranged from 21 to 29 ng/mL. To maintain these concentrations, each calf in the low-status group received a total of 8,600 IU of vitamin D during the trial. As a comparison, $25(OH)D_3$ in calves fed either whole or skimmed milk declined progressively to approximately 10 to 12 ng/mL by 7 d of age (Rajaraman et al., 1997), suggesting that the $25(OH)D_3$ concentrations observed in low- and high-status groups in the current study exceeded values that might be expected in calves fed colostrum and milk only. The markedly lower plasma $25(OH)D_3$ concentrations in calves fed colostrum and milk versus those in calves in the current study and those fed commercial milk replacers (Nonnecke et al., 2007) suggest that the amount of vitamin D added to calf milk replacers needs to be reevaluated.

Because $1,25(OH)_2D_3$ is the principal mediator of the physiologic effects of vitamin D, plasma concentrations of $1,25(OH)_2D_3$ and Ca, Mg, and PO_4 were also evaluated. It is generally recognized that blood $1,25(OH)_2D_3$ concentrations change in response to alterations in blood macromineral concentrations rather than blood concentrations of the pro-hormone, $25(OH)D_3$. A classic example in dairy cattle is the progressive increase in $1,25(OH)_2D_3$ concentrations from <20 pg/mL in the nonpregnant animal to 20 to 50 pg/mL in late gestation and >100 pg/mL at the initiation of lactation in response to increased Ca demand. Once Ca homeostasis is reestablished during lactation, concentrations drop to 0 to 20 pg/mL (Reinhardt et al., 1988). Plasma $1,25(OH)_2D_3$ concentrations in low- and high-status vitamin D calves are shown in Figure 1b. The treatment × time interaction was not significant ($P = 0.91$) for this variable, suggesting that parenteral administration of $25(OH)D_3$, although influencing blood $25(OH)D_3$ concentrations, did not affect $1,25(OH)_2D_3$ concentrations. Results from the correlation analysis indicating that the association between plasma $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations was not significant (r = 0.196, $P = 0.53$, n = 115) confirmed this assumption. Blood $1,25(OH)_2D_3$ concentrations in all calves were greater ($P < 0.01$) at d 0 than at any subsequent time during the 28-d study. During this period, concentrations ranged from 154 pg/mL on d 0 to 56 pg/mL on d 21. These results are similar to those reported by Rajaraman et al. (1997) indicating an age-related decrease in plasma $1,25(OH)_2D_3$ concentrations in preruminant calves beginning at approximately 2 to 4 d of age. Interestingly, several reports have indicated that circulating $1,25(OH)_2D_3$ levels in calves (Naito et al., 1983; Rajaraman et al., 1997) and human infants (Barlet et al., 1981) increase significantly within the first 48 h postpartum, possibly because of synthesis of $1,25(OH)_2D_3$ as a prerequisite for intestinal absorption of calcium and phosphorous (Steichen et al., 1980). Because calves in the current study were more than 2 d of age at the beginning of this study, this early increase in $1,25(OH)_2D_3$ was not seen.

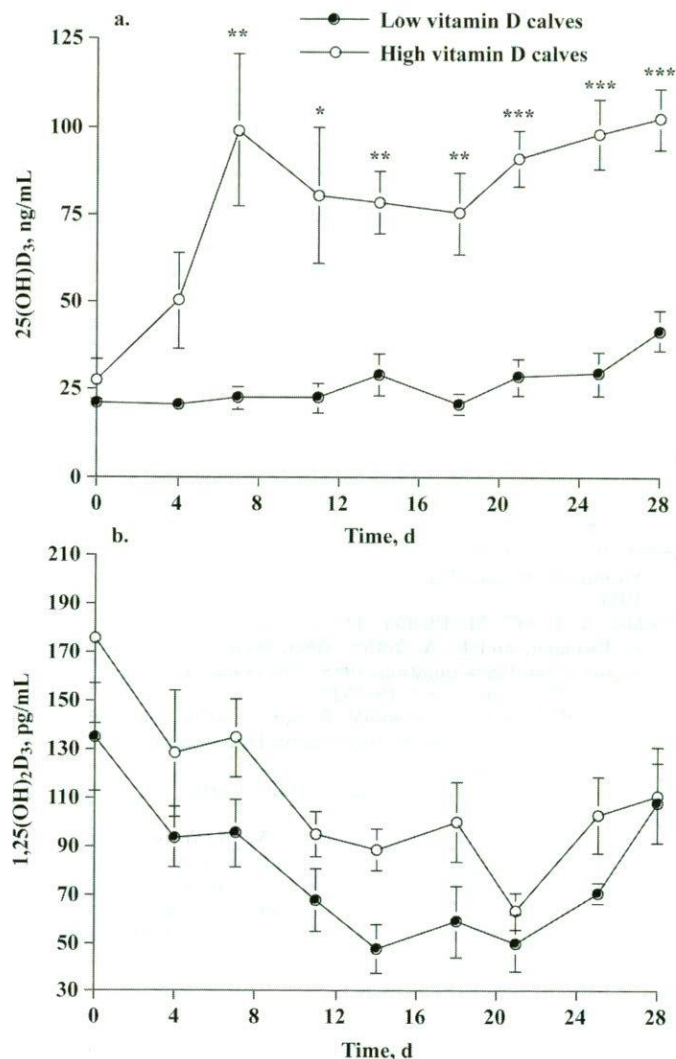Calcium, Mg, and PO_4 are essential for normal function of many tissues and physiologic processes in dairy

Figure 1. Plasma 25-hydroxyvitamin D_3 [25(OH)D_3; panel a] and 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2D_3$; panel b] concentrations in preruminant calves administered 25(OH)D_3 subcutaneously at varying intervals during a 28-d study period with the goal of establishing a low vitamin D status group (n = 6 calves) and high vitamin D status (n = 7) group. Mean plasma 25(OH)D_3 concentrations in low- (27 ng/mL) and high-status (78 ng/mL) groups differed ($P < 0.05$) during the period. Treatment means that differed at a specific time are indicated by *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.
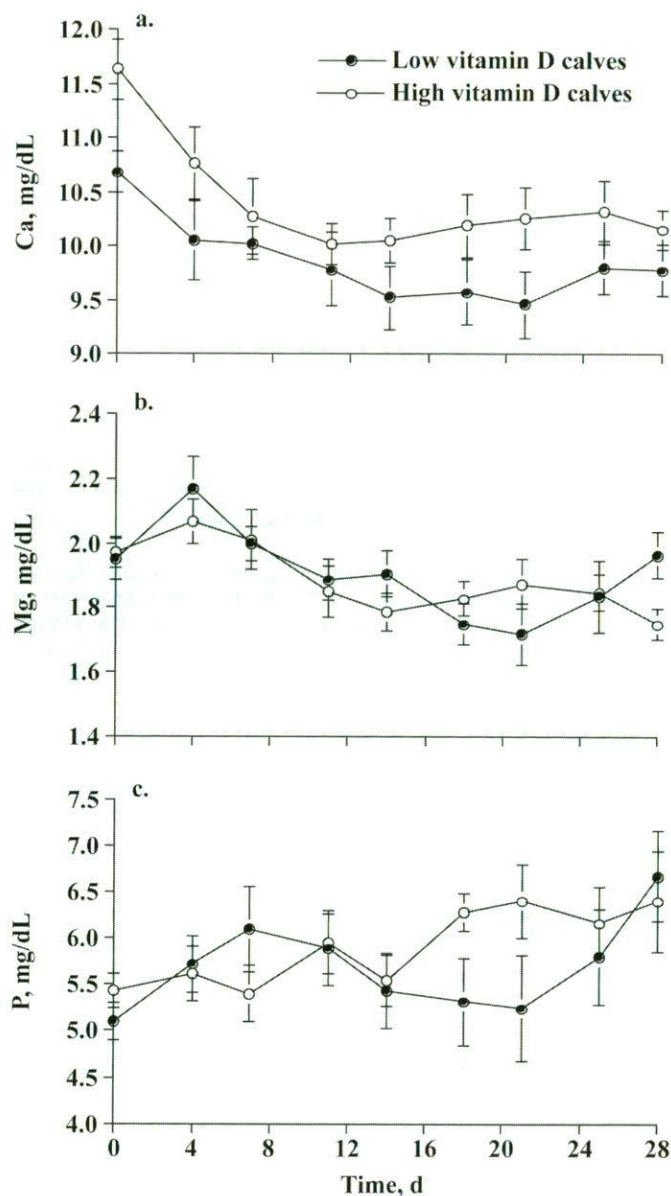

Figure 2. Plasma calcium (panel a), magnesium (panel b), and phosphorous (panel c) concentrations in preruminant calves administered 25-hydroxyvitamin D_3 subcutaneously during a 28-d study period with the goal of establishing a low vitamin D status group (n = 6) and a high vitamin D status group (n = 7).

cattle (Reinhardt et al., 1988; Goff, 2004). Body pools of these macrominerals are regulated by several hormones, including 1,25(OH)$_2D_3$. Although mean plasma 25(OH)D_3 concentrations in low- and high-status calves differed markedly from d 7 through d 28 as a consequence of repeated administration of 1,25(OH)$_2D_3$ (Figure 1a), circulating Ca (Figure 2a), Mg (Figure 2b), and PO$_4$ (Figure 2c) concentrations were unaffected. In all cases, the treatment effects and treatment × time interactions were not significant ($P > 0.05$). These results are not surprising given that 1,25(OH)$_2D_3$ concentra-

tions were also unaffected by the amount of 25(OH)D_3 administered. Blood 1,25(OH)$_2D_3$ concentrations were associated with blood Ca (r = 0.48, $P < 0.0001$) concentrations (Figure 3), but not blood Mg (r = 0.12, $P = 0.20$) or PO$_4$ (r = 0.05, $P = 0.58$) concentrations.

Considering all calves, blood calcium concentrations decreased ($P < 0.001$) with age and ranged from 11.2 mg/dL on d 0 to 9.8 mg/dL on d 14, within the normal range for young calves (NRC, 2001). Blood Mg concentrations varied significantly ($P < 0.0001$) during the
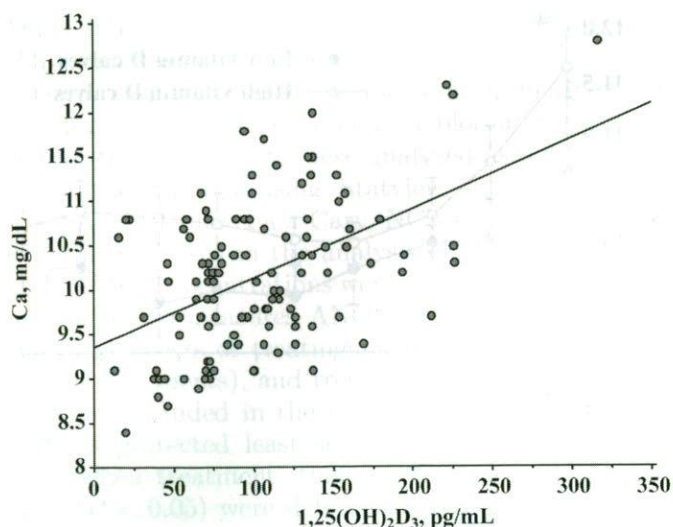
Figure 3. Regression plot describing the association between plasma 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] and plasma calcium concentrations in all calves during the 28-d study period. The relationship between these variables is described by Y = 193.5 + 29.1x, R^2 = 0.227, n = 117.

study and ranged from 2.12 mg/dL on d 4 to 1.80 mg/dL on d 18. Although a normal range for plasma Mg has not been established for preruminant calves, these values fall within the normal range (1.8 to 2.4 mg/dL) for the adult dairy cow (NRC, 2001). Phosphorous concentrations in dairy cattle normally range from 4 to 8 mg/dL, although this range can be higher (6 to 8 mg/dL) in growing cattle (NRC, 2001). Blood PO_4 concentrations for calves in the current study did not change ($P = 0.11$) with time, ranging from 5.3 mg/dL on d 0 to 6.3 mg/dL on d 28. Plasma macromineral concentrations in the current study are comparable to those in calves fed standard and intensified milk replacers (Nonnecke et al., 2007).

In conclusion, results indicate that vitamin D status of the preruminant calf can be controlled using parenteral administrations of 25(OH)D$_3$. The high and low vitamin D–status groups generated using this approach, although differing with regard to plasma 25(OH)D$_3$ levels, would not be considered to be outside normal ranges for either 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$. The groups also did not differ with regard to plasma Ca, Mg, or PO_4 concentrations. By modifying 25(OH)D$_3$ dosage and frequency of administration, this model could be used to examine not only the effects of vitamin D deficiency on mineral metabolism but also the effects of subclinical deficiency on immune regulation. Finally, these results and those from recent studies suggest that levels of vitamin D in commercial milk replacers exceed the requirements of preweaned dairy calves.

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